

Lactose Metabolism in *Streptococcus lactis*: Phosphorylation of Galactose and Glucose Moieties In Vivo

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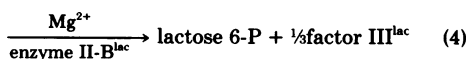
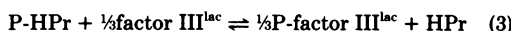
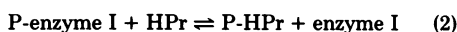
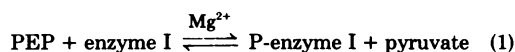
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Starved cells of *Streptococcus lactis* ML₃ grown previously on lactose, galactose, or maltose were devoid of adenosine 5'-triphosphate and contained only three glycolytic intermediates: 3-phosphoglycerate, 2-phosphoglycerate, and phosphoenolpyruvate (PEP). The three metabolites (total concentration, ca. 40 mM) served as the intracellular PEP potential for sugar transport via PEP-dependent phosphotransferase systems. When accumulation of [¹⁴C]lactose by iodoacetate-inhibited starved cells was abolished within 1 s of commencement of transport, a phosphorylated disaccharide was identified by autoradiography. The compound was isolated by ion-exchange (borate) chromatography, and enzymatic analysis showed that the derivative was 6-phosphoryl-*O*- β -D-galactopyranosyl (1 \rightarrow 4')- α -D-glucopyranose (lactose 6-phosphate). After maximum lactose uptake (ca. 15 mM in 15 s) the cells were collected by membrane filtration and extracted with trichloroacetic acid. Neither free nor phosphorylated lactose was detected in cell extracts, but enzymatic analysis revealed high levels of galactose 6-phosphate and glucose 6-phosphate. The starved organisms rapidly accumulated glucose, 2-deoxy-D-glucose, methyl- β -D-thiogalactopyranoside, and *o*-nitrophenyl- β -D-galactopyranoside in phosphorylated form to intracellular concentrations of 32, 32, 42, and 38.5 mM, respectively. In contrast, maximum accumulation of lactose (ca. 15 mM) was only 40 to 50% that of the monosaccharides. From the stoichiometry of PEP-dependent lactose transport and the results of enzymatic analysis, it was concluded that (i) ca. 60% of the PEP potential was utilized via the lactose phosphotransferase system for phosphorylation of the galactosyl moiety of the disaccharide, and (ii) the residual potential (ca. 40%) was consumed during phosphorylation of the glucose moiety.

Group N streptococci (*Streptococcus lactis*, *S. cremoris*, and *S. diacetylactis*) are industrially important microorganisms by virtue of their ability to ferment lactose rapidly and in homo-lactic fashion to lactic acid (24, 25).

The transport and subsequent metabolism of lactose by group N streptococci is believed to occur by a metabolic sequence similar to that proposed for *Staphylococcus aureus* (Fig. 1). In this organism entry and accumulation of lactose is mediated via the phosphoenolpyruvate (PEP): lactose phosphotransferase system (lac-PTS) (11, 18, 31), in which the disaccharide becomes phosphorylated simultaneously with translocation (for a review, see reference 33):



In this multicomponent system the two general proteins, HPr and enzyme I, are both soluble and are synthesized constitutively. The β -galactoside-specific proteins factor III^{lac} and enzyme II-B^{lac} (35-37) are thought to be induced by galactose 6-phosphate (gal 6-P) (31). Hengstenberg et al. (15) reported the accumulation of lactose by *S. aureus* as the monophosphate ester, and the primary hydroxyl group (C-6) of the galactosyl moiety was the suggested site of phosphorylation (lactose 6-phosphate [lac 6-P], reaction 4). Cleavage of lac 6-P by phospho- β -D-galactoside galactohydrolase (19) yields glucose and gal 6-P, which are further metabolized by the Embden-Meyerhof and D-tagatose 6-phosphate pathways (3, 4), respectively.

A similar scheme for lactose fermentation by group N streptococci is suggested by complementation studies (1, 10, 28) of the lac-PTS (28, 29, 40) and by the fact that enzymes of the proposed pathways (5, 20, 30) are present in lactose-grown (and galactose-grown) organisms. However, with the exception of *S. aureus*, lac 6-P has not been isolated from any other micro-

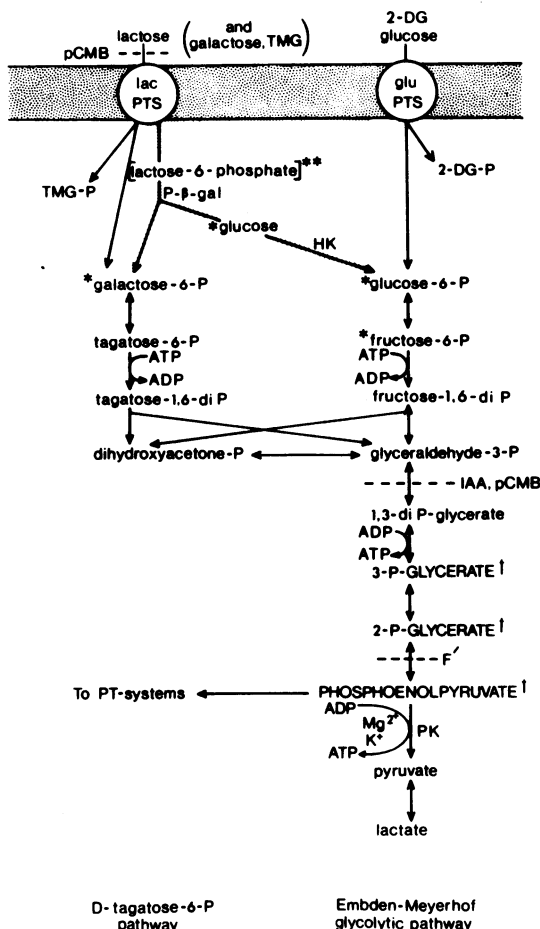


FIG. 1. Probable pathways for lactose and glucose fermentations by *S. aureus* and group *N* streptococci. Daggers (†) indicate compounds comprising the total PEP potential in starved cells of *S. lactis* ML₃; single asterisks (*) indicate intermediates identified and assayed by enzymatic analysis after lactose uptake by IAA-inhibited starved cells of *S. lactis* ML₃; and the double asterisk (**) indicates the first intracellular derivative of PEP-dependent lactose transport in *S. aureus*. Dashed lines show points of inhibition by *p*-chloromercuribenzoate (pCMB), IAA, and fluoride (F⁻). PTS refers to PEP:sugar PTSs. P-β-gal, β-D-Phosphogalactoside galactohydrolase; HK, hexokinase; PK, pyruvate kinase; TMG, methyl-β-D-thiogalactopyranoside; 2-DG, 2-deoxy-D-glucose.

organism, and although frequently inferred (7, 10, 30), the formation of this derivative by group *N* (or other) streptococci has not been reported.

Recent studies in this laboratory (40, 41) showed that (i) starved cells of *S. lactis* ML₃ maintained a PEP potential of ca. 40 mM, and (ii) PTSs were resistant to iodoacetate (IAA). The capacity to dissociate sugar transport from subsequent glycolysis was exploited in this investigation for the isolation of the first intracel-

lular derivative of PEP-dependent lactose transport in *S. lactis*. The results of in vivo experiments suggest that the phosphorylations of the galactosyl and glucosyl moieties of the disaccharide are mediated by separate but highly integrated mechanisms when the PEP potential is used as the endogenous phosphoryl donor.

MATERIALS AND METHODS

Organism. *S. lactis* ML₃ was obtained from the culture collection of the New Zealand Dairy Research Institute.

Culture maintenance and growth of cells. The organism was maintained and grown in a chemically defined medium (39) containing galactose (0.5%, wt/vol) as the fermentable energy source, except where otherwise stated. A homogenous, thick suspension of the starved cells containing 20 to 25 mg (dry weight) of cells per ml was prepared as described previously (39).

Determination of intracellular PEP potential of starved cells by ONPG hydrolysis. Samples of the thick cell suspension (2 to 8 mg [dry weight] of cells) were suspended in 4.8 ml of 0.05 M Tris-maleate buffer (pH 7.0) containing 10 mM IAA. After 10 min of incubation at 30°C, 0.2 ml of 0.01 M *o*-nitrophenyl-β-D-galactopyranoside (ONPG) solution was added to each system, and incubation was continued for an additional 20 min. Subsequently, 1 ml of a 1 M Na₂CO₃ solution was added to each system (final pH, 10.4), and cells were removed by centrifugation (12,000 × *g*, 5 min). The absorbances of the clarified supernatant fluids were determined with a Unicam SP 500 spectrophotometer (Pye Unicam Ltd., Cambridge, England) operated at 420 nm with a 1-cm light path, and *o*-nitrophenol (ONP) formation was determined from a standard curve.

Accumulation of sugars by starved cells. In the standard procedure, ca. 80 to 100 μl of thick cell suspension was added to 9.8 ml of 0.1 M Tris-maleate buffer (pH 7.0) containing 10 mM IAA to obtain a final cell density of 200 μg (dry weight) of cells per ml. After 10 min of incubation ¹⁴C-labeled sugar was added (usually 0.1 mM; specific activity, 0.2 to 0.5 μCi/μmol), and accumulation was followed by using the membrane filtration and liquid scintillation techniques described previously (39). All transport experiments were performed at 30°C. In kinetic studies initial rates of [¹⁴C]lactose uptake were determined after 5 s of incubation.

Preparation of cells containing [¹⁴C]PEP potential. Freshly prepared starved cells were resuspended in 20 ml of 0.1 M Tris-maleate buffer (pH 7.0) containing 1.25 mM [¹⁴C]glucose (0.44 μCi/μmol) at a density of 6 mg (dry weight) of cells per ml. This amount of glucose would be consumed in approximately 2 min (42), but to ensure that starved cells contained only ¹⁴C-labeled PEP potential, the incubation was continued for 10 min. The cells were collected by centrifugation (12,000 × *g*, 1 min), the supernatant fluid was decanted, and the cell pellet was resuspended with 5.5 ml of 0.1 M Tris-maleate buffer, pH 7.0.

Utilization of intracellular [¹⁴C]PEP potential during sugar transport. In these experiments 1-ml

volumes of the previously described ^{14}C -labeled cell suspension (ca. 20 mg [dry weight] of cells) were suspended in 9 ml of 0.1 M Tris-maleate buffer (pH 7.0) containing, when required, 1 mM *p*-chloromercuribenzoate or 10 mM IAA. After 10 min of incubation at 30°C, glucose or lactose (final concentration, 0.5 mM) was added to the appropriate system, and 20 s later the cells were collected by membrane filtration. The cells were extracted as described in a previous report (40). The freeze-dried residues were reconstituted with 200 μl of distilled water, and 10- μl samples were assayed qualitatively by thin-layer chromatography.

Intracellular derivatives from [^{14}C]lactose transport (>15 s). Starved cells were suspended at a density of 1 mg (dry weight) per ml in a series of four flasks, each containing 10 ml of 0.1 M Tris-maleate buffer (pH 7.0) plus 10 mM IAA. After 10 min of preincubation, [^{14}C]lactose (specific activity, 1 $\mu\text{Ci}/\mu\text{mol}$) was added to a final concentration of 0.2 mM, and after 15 s the cells were collected by filtration through a membrane filter (diameter, 47 mm; pore size, 0.8 μm) and transferred immediately to 5 ml of 10% (wt/vol) trichloroacetic acid solution at 0°C. This procedure was repeated with the remaining systems, allowing 25 s, 35 s, and 4 min of incubation, respectively. Cell extracts were prepared as described previously (8, 41), and freeze-dried residues were reconstituted with 0.3 ml of distilled water. Radiolabeled derivatives were separated by thin-layer chromatography (9) on polyethyleneimine-cellulose and identified by autoradiography and co-chromatography with standard compounds (40). Qualitative identifications of free and phosphorylated sugars were confirmed by ion-exchange chromatography (see Fig. 8) and high-voltage electrophoresis (see Fig. 9). In these experiments 0.1 ml of reconstituted extract (ca. 180,000 cpm) was mixed with 0.4 ml of 0.05 M imidazole-hydrochloride buffer, pH 7.5. Alkaline phosphatase (10 U, *Escherichia coli*) was added to a duplicate system. After incubation for 1 h at 25°C, the solutions were transferred to anion-exchange columns (0.6 by 4 cm; Bio-Rad AGI-X4 resin; 100 to 200 mesh; formate form). Free sugars and phosphorylated derivatives were eluted sequentially with distilled water and a solution of 0.5 M ammonium formate in 0.2 M formic acid, respectively (26, 40). Fractions (0.5 ml) were collected, and radioactivity was determined by mixing 50- μl samples from each fraction with 8 ml of scintillation cocktail (100 g of naphthalene plus 5 g of PPO [2,5-diphenyloxazole] per liter of dioxan). A quench correction was applied for samples eluted with the formic acid-ammonium formate solution, and recovery of ^{14}C -labeled material was ca. 100%. Free sugar fractions obtained before (see Fig. 8, peak F₁) and after (peak F₂) alkaline phosphatase treatment were pooled, freeze dried, and reconstituted with 75 μl of distilled water. For high-voltage electrophoresis (4,000 V, 100 mA, 45 min) 15- μl samples were applied to Whatman no. 1 chromatography paper presoaked with 0.05 M sodium borate buffer (pH 10.4) together with 25 μg of sugar standards and 2,3,4,6-tetramethylglucose as an endosmosis marker. Sugars were identified by heating the electropherogram at 120°C after spraying with *p*-aminohippuric-phthalic acid reagent or by autoradiogra-

phy. M_G values for standard sugars were as follows: glucose, 1.0; galactose, 0.94; fructose, 0.88; and lactose, 0.48.

Separation of sugars and sugar phosphates by ion-exchange chromatography. The separation of sugar and sugar-phosphate mixtures by ion-exchange chromatography (as borate complexes) has been described by Khym and Cohn (23), and the modified procedure of Lefebvre et al. (27) was used in this study. Analytical-grade anion-exchange resin (Bio-Rad Laboratories; AGI-X4; 100 to 200 mesh; Cl^- form) was washed successively with 1 N NaOH, 3 N HCl, and finally 0.6 M potassium tetraborate to convert the resin into the borate form. Columns (0.9 by 100 cm) were packed with resin, a sample (0.5 ml) was applied, and elution was performed by using a linear gradient (0.1 to 0.6 M) of freshly prepared ammonium tetraborate. The 0.1 and 0.6 M ammonium tetraborate solutions were prepared by adding 9.9 and 59.4 g of H_3BO_3 , respectively, to ca. 300 ml of distilled water, adjusting to pH 10 by adding aqueous ammonia (specific gravity, 0.89), and finally making up the volume to 400 ml with water. During gradient elution (total volume, 800 ml) fractions of 3.5 ml were collected at a flow rate of ca. 1.3 ml/min, and 0.1-ml volumes of alternate fractions were assayed for ^{14}C . The elution profiles of free sugars and sugar phosphates standards were determined by the anthrone method (2), using 0.5 ml of each fraction, and ketose compounds were confirmed by the procedure of Roe (34).

Isolation of [^{14}C]lactose-phosphate. Starved cells of *S. lactis* ML₃ were suspended at a density of 25 mg (dry weight) of cells per ml in 0.1 M Tris-maleate buffer (pH 7.0) containing 10 mM IAA. Three 1-ml volumes of cell suspension were incubated for 10 min at 30°C, and [^{14}C]lactose was added to each system to a final concentration of 0.32 mM (specific activity, 5 $\mu\text{Ci}/\mu\text{mol}$). Sugar transport was halted after 1 s by injection of 5 ml of 12% (wt/vol) trichloroacetic acid solution at 0°C. The three solutions were maintained at this temperature for ca. 20 min and then centrifuged at 27,000 $\times g$ for 10 min. The clarified supernatant fluids were extracted with five 6-ml volumes of water-saturated diethyl ether, pooled, adjusted to pH 7.0 by the addition of K_2CO_3 , and freeze dried. The residue (7.6×10^6 cpm) was reconstituted to 0.7 ml with distilled water, and 0.5 ml of the solution was applied to the ion-exchange column (borate form) and eluted with ammonium tetraborate. Fractions containing presumptive [^{14}C]lactose-phosphate (ca. 1.48×10^6 cpm) were pooled and freeze dried. Ammonium tetraborate was removed (as methyl borate) by repeated distillation with anhydrous methanol (six times, 50 ml each) in vacuo (32, 44).

The residue was dissolved in water, residual ammonium ions were removed by ion-exchange (H^+) chromatography, and the eluent was taken to dryness by rotary evaporation. After a final distillation with methanol, the residue (ca. 1.35×10^6 cpm) was taken up with 0.55 ml of distilled water.

Enzymatic analyses. Intracellular metabolites and free sugars present in cell extracts were assayed enzymatically by using fluorescence spectrophotometry as described previously (40, 41). All assays were conducted at 25°C, using 10 to 30 μl of cell extract.

Glucose 6-phosphate (G6P) and fructose 6-phosphate (F6P) were assayed by the sequential addition of glucose 6-phosphate dehydrogenase (D-glucose-6-phosphate:NADP 1-oxidoreductase, EC 1.1.1.49; bakers' yeast, 4 U) and phosphoglucose isomerase (D-glucose-6-phosphate ketol-isomerase, EC 5.3.1.9; yeast, 4 U) to the same cuvette containing 5 μ M NADP and 5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$. The amount of time required for completion of each reaction step was ca. 5 min.

Glucose was determined by the addition of glucose-6-phosphate dehydrogenase (1.2 U) and hexokinase (ATP:D-hexose-6-phosphotransferase, EC 2.7.1.1; yeast, 4.4 U) to the assay system which contained 0.05 M imidazole-hydrochloride buffer (pH 7.5), 4 μ M ATP, 5 μ M NADP, and 5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$.

ATP was assayed as described above for glucose, except that ATP was omitted and 0.1 mM glucose was included in the assay system (14).

Galactose and gal 6-P were determined by a fluorimetric modification of the method of Grassl (13). Free sugar was determined by using the galactose dehydrogenase (D-galactose:NAD 1-oxidoreductase; EC 1.1.1.48)-NAD coupled indicator system. The basal assay system contained 0.05 M imidazole-hydrochloride buffer (pH 7.5), 10 μ M NAD, and galactose dehydrogenase (*Pseudomonas fluorescens*; 0.4 U). The reaction time was 60 min at 25°C. Gal 6-P was measured as the free sugar after incubation with alkaline phosphatase (EC 3.1.3.1, *E. coli*).

Paper chromatography. Separation of sugars and phosphorylated derivatives in cell extracts was performed by ascending chromatography (20 h) on Whatman no. 1 paper, using the solvent I system (pH 5.5) described by Wawszkiewicz (43). Phosphate compounds were identified as white spots on a pink background upon dipping the chromatogram in (i) a solution containing 50 mg of FeCl_3 , 3 ml of 0.3 M HCl, and 97 ml of acetone, followed by (ii) 1.25% (wt/vol) sulfosalicylic acid in acetone. Free sugars were also separated by descending chromatography on Whatman no. 1 paper (24 h), using a butyl acetate-ethanol-pyridine-water (8:2:2:1, vol/vol) solvent. Sugars were located by spraying the dry chromatogram with alkaline silver nitrate solution. Radioactive compounds were detected by autoradiography.

Intracellular solute concentrations. The millimolar concentrations of intracellular metabolites were calculated on the assumption that for *S. lactis* ML₃ 1 g (dry weight) of cells was equivalent to 1.67 ml of intracellular (protoplast) fluid (39). A similar value (ca. 1.5 ml) has been reported by Kashket and Wilson (21) for *S. lactis* 7962.

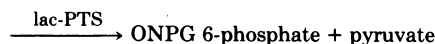
Reagents. Chemicals and enzymes were purchased from the Sigma Chemical Co., St. Louis, Mo. D-[U-¹⁴C]glucose and [D-glucose-1-¹⁴C]lactose were from the Radiochemical Centre, Amersham, England, and [methyl-¹⁴C]- β -D-thiogalactopyranoside was purchased from New England Nuclear Corp., Boston, Mass. Precoated layers of polyethyleneimine-cellulose were obtained from Macherey, Nagel and Co., Duren, Germany.

RESULTS

Intracellular PEP potential of starved cells. By enzymatic analysis, starved cells of *S.*

lactis ML₃ were found to contain an intracellular pool of three glycolytic intermediates, namely 3-phosphoglycerate (28.9 mM), 2-phosphoglycerate (5.3 mM), and PEP (11.3 mM), which were equivalent to a PEP generating potential of ca. 45 mM (40). A similar estimate (ca. 38.5 mM) for this potential was obtained indirectly in this study by using the chromogenic lactose analog ONPG, as shown by the following equations:

ONPG + PEP



ONPG 6-phosphate



(P- β -gal is phospho- β -D-galactoside galactohydrolase.) Cells of *S. lactis* ML₃ do not contain β -galactosidase, and ONP formation should therefore reflect the PEP potential, provided that there is a tight coupling of the reactions (22). It was found (Fig. 2A) that ONP production by IAA-inhibited starved cells was directly proportional to cell density (64.3 μ mol/g [dry weight] of cells). From this value and by assuming an intracellular (protoplast) volume of 1.67 ml/g, the calculated PEP potential was approximately 38.5 mM. In the presence of F⁻ (an inhibitor of enolase in vivo) (41), the formation of ONP was reduced by ca. 76% (15.5 μ mol/g [dry weight] of cells), which is indicative of an available PEP potential of only 9.3 mM. A theoretical reduction of 75% would have been predicted from the enzymatic analysis data (see above). No liberation of ONP occurred when cells were exposed to *p*-chloromercuribenzoate, suggesting inhibition of the lac-PTS (Fig. 2A).

Stoichiometry of PEP-dependent sugar accumulation. *S. lactis* ML₃ accumulated glucose and methyl- β -D-thiogalactopyranoside to maximum intracellular concentrations of 32 and 42 mM, respectively (Fig. 2B). Under similar conditions 2-deoxy-D-glucose was accumulated by starved cells to a maximum of ca. 32 mM within 20 s (40). These data suggested a stoichiometric ratio of ca. 0.8 to 1.0 for monosaccharide accumulated/PEP potential utilized by the appropriate PTS. Separate experiments showed that the lac-PTS displayed a high affinity for the disaccharide (K_m , \approx 21 μ M; V_{max} , \approx 150 μ mol/g [dry weight] of cells per min). Maximum accumulation of lactose (ca. 15 mM) was attained within 10 to 15 s (Fig. 2B), but after this time rapid efflux of intracellular material occurred, and only 40% of the radioactivity initially present (at 15 s) remained within the cells after 4 min of incubation. Maximum uptake of [¹⁴C]-lactose was only 40 to 50% that of the monosac-

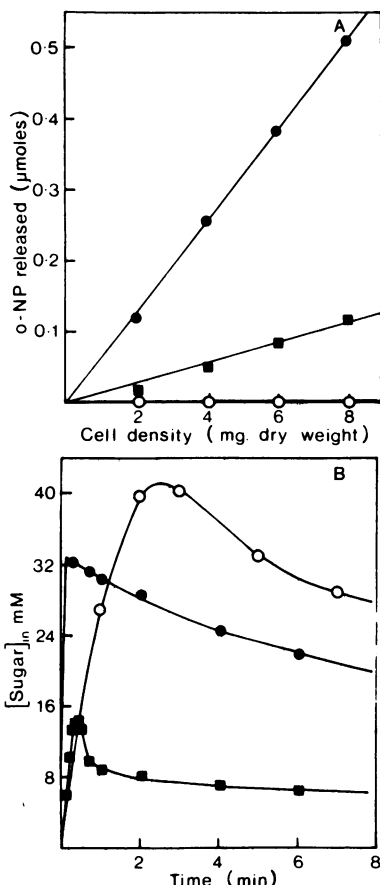


FIG. 2. (A) Effects of inhibitors upon ONPG hydrolysis by starved cells of *S. lactis* ML₃. The cells were suspended at the densities shown in 5 ml of 0.05 M Tris-maleate buffer (pH 7.0) containing 0.4 mM ONPG and the inhibitors at the designated concentrations. Symbols: ●, control (10 mM IAA); ■, 2 mM NaF; ○, 1 mM p-chloromercuribenzoate. Other conditions were as described in the text. (B) Accumulation of sugars by starved cells of *S. lactis* ML₃. Cells were suspended at a density of 200 μg (dry weight) per ml in 0.1 M Tris-maleate buffer (pH 7.0) containing 10 mM IAA. After 10 min of preincubation the following sugars were added to the appropriate system: 0.1 mM [¹⁴C]glucose (●); [methyl-¹⁴C]-β-D-thiogalactopyranoside (○); and 0.1 mM [¹⁴C]lactose (■). The specific activity of all sugars was 0.2 μCi/μmol.

charides. This result suggested that only one-half of the available PEP potential had been utilized for lactose translocation, but as Fig. 3 shows, this was not the case.

Accumulation of sugars and utilization of PEP potential. Starved cells of *S. lactis* ML₃ were prepared containing [¹⁴C]PEP potential (see above). Autoradiography by thin-layer chromatography showed that an extract pre-

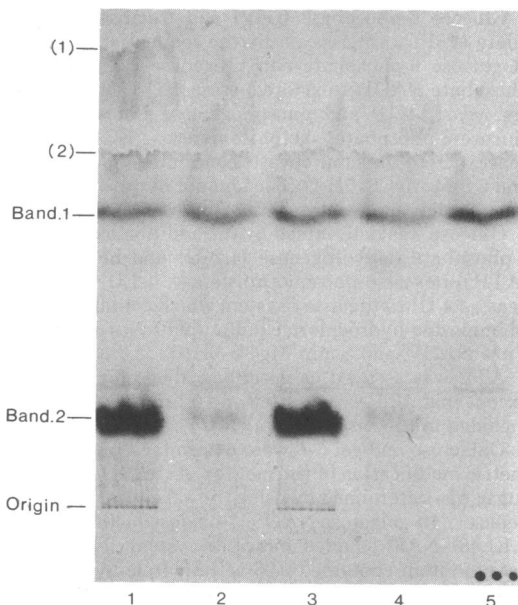


FIG. 3. Utilization of total intracellular [¹⁴C]PEP potential during uptake of glucose and lactose by intact cells of *S. lactis* ML₃. Cells containing [¹⁴C]-labeled PEP potential were suspended at a density of 2 mg (dry weight) per ml in 0.1 M Tris-maleate buffer (pH 7.0) containing 10 mM IAA (control) (lane 1), 0.5 mM lactose plus 10 mM IAA (lane 2), 0.5 mM lactose plus 1 mM p-chloromercuribenzoate (lane 3), 0.5 mM glucose plus 10 mM IAA (lane 4), and 0.5 mM glucose plus 1 mM p-chloromercuribenzoate (lane 5). After 20 s cells were collected by filtration, extracts were prepared, and samples were assayed by autoradiography. Band 1, [¹⁴C]lactic acid; band 2, radioactive mixture of PEP potential intermediates. Solvent fronts (1) and (2) refer to water and lithium chloride-formic acid, respectively.

pared from such cells contained two major bands of radioactivity (Fig. 3, lane 1); band 1 was identified as [¹⁴C]lactic acid, and band 2 contained the three metabolites comprising the [¹⁴C]PEP potential. Maximum accumulation of lactose and glucose by IAA-inhibited starved cells resulted in total utilization of the [¹⁴C]PEP potential (Fig. 3, lanes 2 and 4, respectively). It is of interest to note that p-chloromercuribenzoate selectively inhibited the lac-PTS (Fig. 3, lane 3), confirming previous findings (Fig. 2A).

Intracellular derivatives formed within 1 s of commencement of [¹⁴C]lactose transport. Transport of [¹⁴C]lactose by IAA-inhibited starved cells was arrested within 1 s of commencement by direct injection of trichloroacetic acid solution into the incubation medium (see above). The complete system was extracted, and five radioactive compounds were detected by paper chromatography (Fig. 4, lane 2). The ma-

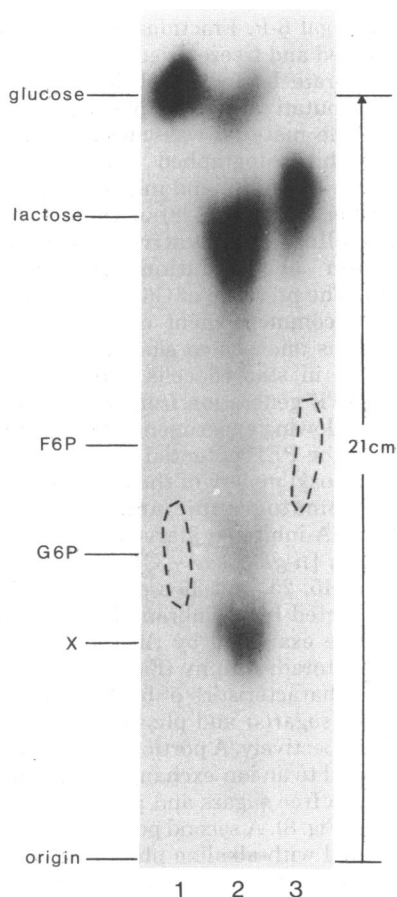


FIG. 4. Autoradiographic identification of metabolites formed by *S. lactis* ML₃ after ca. 1 s of incubation with [¹⁴C]lactose. The complete incubation medium was extracted, and conditions for ascending paper chromatography were as described in the text. Sugar-phosphate standards (dotted areas) were located after autoradiography. Lane 1, [¹⁴C]glucose (20,000 cpm) and G6P (0.25 μ mol); lane 2, 8 μ l (ca. 70,000 cpm) of reconstituted cell extract; lane 3, [¹⁴C]lactose (20,000 cpm) and F6P (0.25 μ mol).

major component, [¹⁴C]lactose (60%), was derived from the medium, and the four remaining compounds were extracted from within the cells; these four were [¹⁴C]glucose (20%), [¹⁴C]G6P (4%), [¹⁴C]F6P (1%), and an unidentified component X, which constituted 15% of the total radioactivity of the extract.

Identification of compound X. Chromatographic migration of the unidentified material X (Fig. 4) suggested an anionic (phosphate?) compound. Phosphorylated materials in the extract were therefore separated from free sugars as described below (see Fig. 8). Formic acid and ammonium formate were removed in vacuo, and the residue was reconstituted with 200 μ l of

distilled water. Samples (40 μ l) were incubated with (i) alkaline phosphatase and (ii) alkaline phosphatase plus β -galactosidase. Alkaline phosphatase treatment produced two radioactive components corresponding to [¹⁴C]glucose (30%) and [¹⁴C]lactose (70%) (Fig. 5, lane 1). After incubation with both enzymes, more than 91% of the total radioactivity was recovered as [¹⁴C]glucose (Fig. 5, lane 2). After alkaline phosphatase treatment, 4.5 nmol of glucose (from G6P) and 11.4 nmol of galactose (from gal 6-P) were found in the assay system by enzymatic analysis. The combined effects of alkaline phosphatase plus β -galactosidase yielded 9.5 nmol of glucose and 16.1 nmol of galactose. Subtraction of the analytical data showed that the lactose derivative had a composition of 5 nmol of glucose and 4.7 nmol of gal 6-P. Derivative X (Fig. 4) was therefore a lactose monophosphate in which phosphorylation occurred on the galactosyl moiety (i.e., lac 6-P).

Isolation of lactose phosphate by ion-exchange chromatography. IAA-inhibited starved cells were incubated for 1 s with [¹⁴C]lactose and extracted as described above. A portion of the extract was fractionated by ion-exchange chromatography (Fig. 6B). Approximately 93% of the radioactivity was recovered in four major peaks: lactose (42%), glucose (20%), G6P (9%), and a derivative eluted at ca. 0.24 M ammonium tetraborate and presumed to be lac-

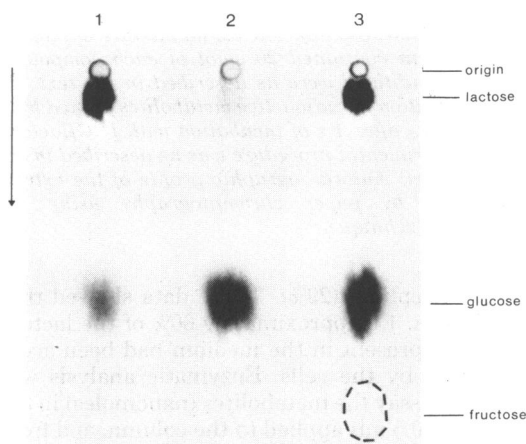


FIG. 5. Autoradiographic identification of intracellular ¹⁴C-labeled sugar phosphates formed ca. 1 s after [¹⁴C]lactose uptake. The preparation of the sugar-phosphate fraction and the method of descending paper chromatography were as described in the text. Lane 1, sugar-phosphate incubated with alkaline phosphatase; lane 2, sugar-phosphate incubated with alkaline phosphatase plus β -galactosidase; lane 3, standard mixture of [¹⁴C]lactose, [¹⁴C]glucose (both 20,000 cpm), and unlabeled fructose (0.25 μ mol).

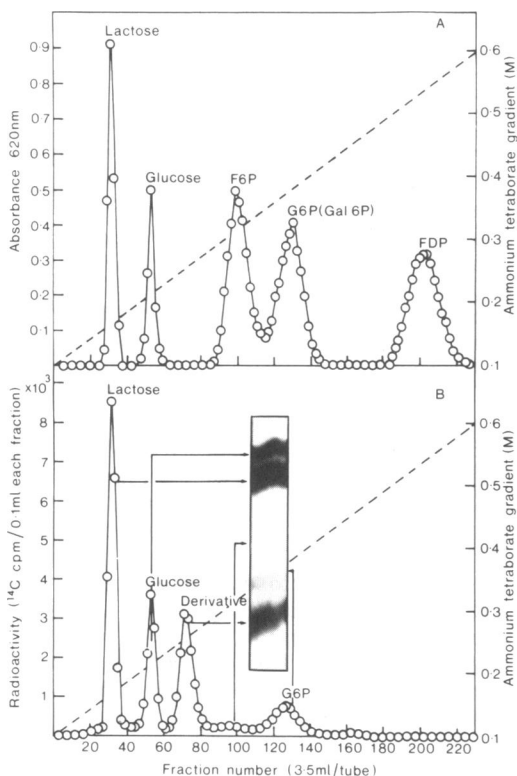


FIG. 6. (A) Separation of a mixture of standard sugars and sugar phosphates by ion-exchange (borate) chromatography. The broken line represents the gradient from 0.1 to 0.6 M ammonium tetraborate (total volume, 800 ml). The 0.5-ml mixture applied to the column contained 20 μ mol of each compound. Other conditions were as described in the text. (B) Identification of radioactive metabolites formed by *S. lactis* ML₃ after 1 s of incubation with [¹⁴C]lactose. The experimental procedure was as described in the text. (Inset) Autoradiographic profile of the extract obtained by paper chromatography using the "wedge" technique.

tose-phosphate (29%). These data showed that within ca. 1 s approximately 60% of the lactose initially present in the medium had been accumulated by the cells. Enzymatic analysis was used to assay the metabolites (nanomoles) in the sample (0.5 ml) applied to the column, and from the known cell density an approximate value was obtained for the intracellular concentration of each metabolite within 1 s of incubation; these values were: [¹⁴C]lactose phosphate, 146 nmol (2.3 mM); [¹⁴C]glucose, 122 nmol (1.9 mM); [¹⁴C]G6P, 156 nmol (2.5 mM); [¹⁴C]F6P, trace amount only; galactose, 14 nmol (0.2 mM); and gal 6-P, 257 nmol (4.1 mM). It should be noted that the total concentration of glucose plus G6P was almost the same as the intracellular concen-

tration of gal 6-P. Fractions 64 to 82 (Fig. 6B) were pooled and freeze dried, and after removal of tetraborate by methanol distillation, ca. 80 nmol of putative lactose phosphate was obtained. The material was chromatographically pure, cochromatographed with α -lactose 1'-phosphate (R_f , 0.14), and migrated more slowly than either G6P (R_f , 0.28) or gal 6-P (R_f , 0.25).

Intracellular derivatives formed after maximum accumulation of [¹⁴C]lactose (>15 s). The presence of G6P in starved cells at 1 s after commencement of lactose transport (Fig. 4) was unexpected since (i) ATP was not detectable in starved cells and (ii) IAA prevented ATP generation from glycolysis (Table 1). The following experiments indicated participation of the PEP potential in phosphorylation of the glucosyl moiety of the disaccharide.

(i) Chromatographic analysis of cell extracts. IAA-inhibited starved cells were incubated with [¹⁴C]lactose, and at intervals of 15, 25, and 35 s and 4 min the cells were collected by membrane filtration and extracts were examined by thin-layer chromatographic autoradiography (Fig. 7). The chromatographic characteristics of bands A and B indicated free sugar(s) and phosphorylated derivative(s), respectively. A portion of the 15-s extract was applied to an ion-exchange column and separated into free sugars and phosphorylated derivatives (Fig. 8). A second portion of the extract was treated with alkaline phosphatase. The resultant chromatographic profile (Fig. 8) showed a threefold increase in free sugar(s) and the

TABLE 1. Intracellular ATP concentrations in intact cells of *S. lactis* ML₃ maintained under different physiological conditions

Physiological state ^a	Intracellular ATP concn (mM)	
	Expt 1	Expt 2
Starved cells	ND ^b	ND
Glycolyzing cells	5.67	6.50
Glycolyzing cells (+IAA)	0.23	0.57
Glycolyzing cells (+ <i>p</i> -chloromercuribenzoate)	0.79	0.68

^a Incubation systems and experimental protocol were as described in the text. IAA and *p*-chloromercuribenzoate were added to glycolyzing cell suspensions to final concentrations of 10 and 1 mM, respectively. The cells were collected by membrane filtration at 30 s after addition of the —SH reagents; cell extracts were prepared and assayed for ATP as described in the text.

^b The sensitivity of the fluorimetric assay for ATP was 2×10^{-11} mol, which if present in 30 μ l of cell extract (see text) would be equivalent to an intracellular nucleotide concentration of ca. 0.1 mM. ND, Not detectable.

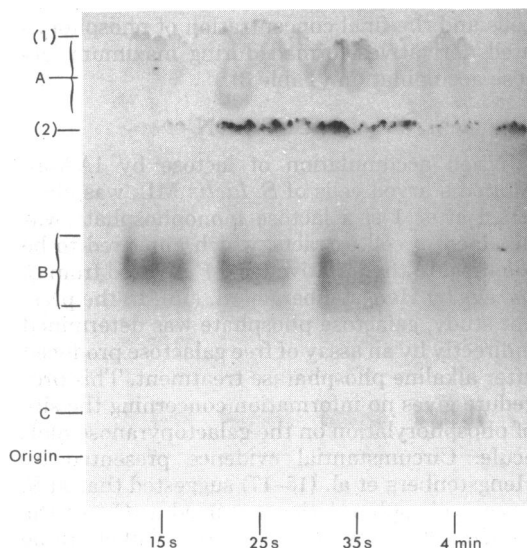


FIG. 7. Time course autoradiographic analysis by thin-layer chromatography of intracellular metabolites formed during [^{14}C]lactose accumulation by IAA-inhibited starved cells of *S. lactis* ML₃. The cells were suspended at a density of 1 mg (dry weight) per ml in 0.1 M Tris-maleate buffer (pH 7.0) containing 10 mM IAA. After 10 min of incubation, 0.2 mM [^{14}C]lactose (1 $\mu\text{Ci}/\mu\text{mol}$) was added to the system. After 15, 25, and 35 s and 4 min of incubation the cells from 10 ml of suspension were collected by membrane filtration and extracts were prepared. Bands A and B contained free sugars and phosphorylated derivatives, respectively (see text); band C indicates trace amounts of fructose 1,6-bisphosphate. Intracellular concentrations of sugar phosphates (determined by enzymatic analysis) after 15, 25, and 35 s and 4 min were 10.8, 10.9, 8.8, and 4.6 mM for G6P and 21.3, 23.6, 23.6, and 9.5 mM for gal 6-P. The numbers 1 and 2 refer to water and lithium chloride-formic acid solvent fronts, respectively. (The dark region associated with solvent front 2 is an artifact caused by adherence of the layer to the X-ray film during exposure.)

absence of phosphorylated compounds. Free sugars present in the extract before and after alkaline phosphatase treatment (Fig. 8, peaks F_1 and F_2 , respectively) were quantitatively identified by high-voltage electrophoresis and autoradiography (Fig. 9). The results showed that peak F_1 (equivalent to Fig. 7, band A) contained [^{14}C]glucose (86%) and [^{14}C]lactose (14%). The [^{14}C]lactose used for this experiment contained <1% [^{14}C]glucose as contaminant; therefore, the [^{14}C]glucose detected must have been produced within the cells during lactose uptake. The F_2 peak contained [^{14}C]glucose, [^{14}C]fructose, and an unidentified component. The composition of band B (Fig. 7) was determined by the difference between peak F_2 and peak F_1 ; this band con-

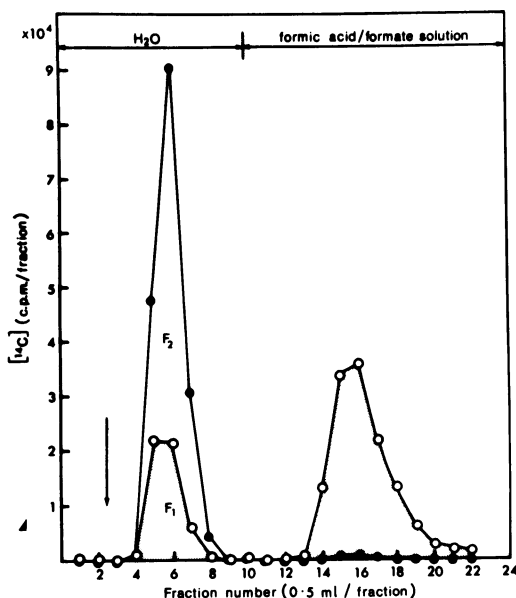


FIG. 8. Ion-exchange chromatography of 15-s cell extract (Fig. 7) before (○) and after (●) alkaline phosphatase treatment. Experimental conditions were as described in the text; fractions 4 through 11 contained free sugars, and fractions 13 through 22 contained phosphorylated compounds. Peaks designated F_1 and F_2 represent free sugars in the extract before and after alkaline phosphatase treatment, respectively.

tained G6P (60%), F6P (20%), and an unknown phosphorylated derivative (ca. 20%). It is important to note that the amount of [^{14}C]lactose present in peak F_2 was not significantly greater than that in peak F_1 . This result, which demonstrated the absence of lac 6-P in the 15-s cell extract, was confirmed by using the 25-s, 35-s and 4-min extracts.

(ii) **Enzymatic analysis of cell extracts.** Enzymatic analysis confirmed the presence of high levels of G6P and gal 6-P in the four cell extracts (Fig. 7), but the intracellular concentrations of hexose phosphates decreased with increased incubation period (Fig. 2A). Data obtained previously suggest that the observed decrease is due to dephosphorylation and efflux of the free sugars (40). In subsequent experiments *S. lactis* ML₃ was grown on galactose, lactose, or maltose, and the starved cells were incubated with [^{14}C]lactose for 10 s before filtration and extraction. Enzymatic analysis (Table 2) showed that in each case gal 6-P, G6P, and F6P were the predominant intracellular derivatives. Glucose, galactose, and fructose 1,6-bisphosphate were also present, but lac 6-P was not detected. In these studies there was a close correlation between the initial PEP potential of the starved

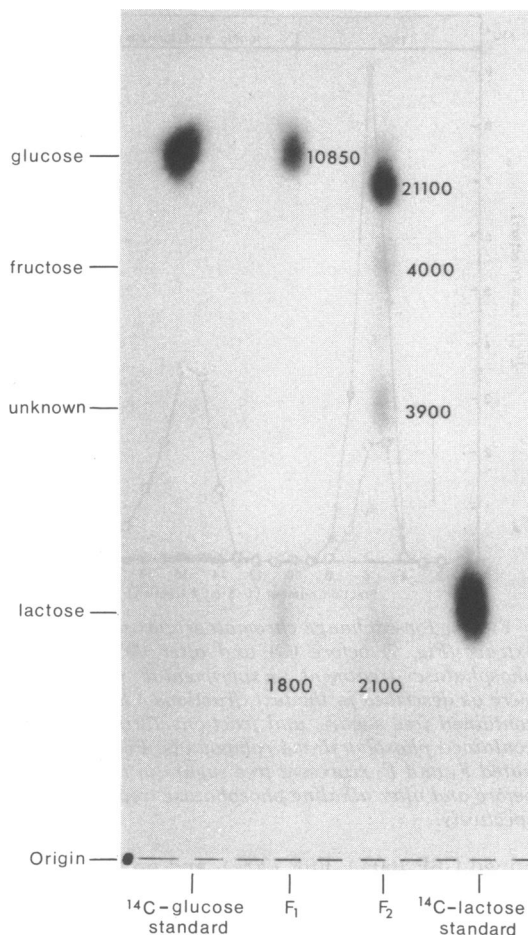


FIG. 9. Identification of free sugars present in peaks F_1 and F_2 (Fig. 8) by high-voltage electrophoresis and autoradiography. Numbers refer to ^{14}C counts per minute after elution of the spots and counting by liquid scintillation.

cells and the final concentration of phosphorylated derivatives formed during maximum lactose accumulation (Table 3).

DISCUSSION

When accumulation of lactose by IAA-inhibited starved cells of *S. lactis* ML₃ was abolished after 1 s, a lactose monophosphate was detected in cell extracts, which appeared to be identical to the putative lac 6-P isolated from *S. aureus* by Hengstenberg et al. (15). In the present study, galactose phosphate was determined indirectly by an assay of free galactose produced after alkaline phosphatase treatment. This procedure gives no information concerning the site of phosphorylation on the galactopyranose molecule. Circumstantial evidence presented by Hengstenberg et al. (15–17) suggested that in *S. aureus* phosphorylation occurred at C-6 of the galactosyl moiety, and this is most likely to be the case in the derivative prepared from *S. lactis* ML₃. Lac 6-P has not been chemically synthesized and is not commercially available. However, lactose 1'-phosphate (phosphorylation at

TABLE 3. Correlation between initial PEP potential *in vivo* and enzymatically determined concentrations of phosphorylated metabolites formed during maximum lactose uptake by IAA-inhibited starved cells of *S. lactis* ML₃

Growth sugar	PEP potential of IAA-inhibited starved cells (mM) ^a	Total intracellular concn of phosphorylated metabolites (mM) ^b
Galactose	38.5	37.8
Lactose	32.2	40.4
Maltose	45.6	50.5

^a Determined by ONPG hydrolysis method.

^b Data obtained from Table 2.

TABLE 2. Intracellular concentrations of metabolites formed after maximum accumulation of lactose by IAA-inhibited starved cells of *S. lactis* ML₃

Growth sugar	Intracellular concn of metabolite (mM) ^a						
	Glucose	G6P	F6P	Fructose 1,6-bisphosphate	Triose phosphate	Galactose	Gal 6-P
Galactose	6.64 ± 1.12	9.08 ± 0.51	2.01 ± 0.38	Trace (ca. 0.2) ^b	ND ^c	2.82 ± 0.21	26.70 ± 3.28
Lactose	3.29 ± 0.80	8.78 ± 0.73	2.00 ± 0.93	1.20 ± 0.00	0.44 ± 0.21	1.99 ± 0.34	26.79 ± 0.89
Maltose ^d	2.06 ± 0.42	13.85 ± 0.90	4.37 ± 0.44	1.70 ± 0.39	0.83 ± 0.23	0.67 ± 0.46	27.28 ± 0.72

^a Starved cells of *S. lactis* ML₃ (grown previously on either galactose, lactose, or maltose) were initially devoid of these metabolites. No other glycolytic intermediates were detectable in cell extracts by either autoradiographic or enzymatic analysis. The results represent means and standard deviations from duplicate analyses of cell extracts obtained from three separate experiments. The incubation period for lactose uptake was ca. 10 s before membrane filtration. The experimental procedure, preparation of cell extracts, and methods of enzymatic analysis were as described in the text.

^b Detectable by autoradiography (see Fig. 7).

^c ND, Not detectable by enzymatic analysis.

^d Gal 6-P is the probable inducer of the lac-PTS (31). It is not apparent why induction of this system should occur during growth on maltose (4-*O*-β-D-glucopyranosyl-D-glucose), but Cords and McKay (10) reported similar findings with *S. lactis* C₂. Maltose transport in *S. lactis*, as in *S. aureus* (6), occurs via a non-PTS system (26; Thompson, unpublished data).

the anomeric C-1 center of the glucose moiety) has been prepared enzymatically (12) and can also be obtained from commercial sources. The lactose derivative isolated from *S. lactis* ML₃ cochromatographed with the lactose 1'-phosphate isomer.

The PEP potential of starved cells, determined by two independent methods, was ca. 40 mM. Transport studies conducted in the presence of IAA suggested a ratio close to unity for the stoichiometry of maximum uptake of monosaccharide/PEP potential utilized. Maximum accumulation of lactose (ca. 15 mM) was approximately one-half that of monosaccharides, and based on the ratio of 1 mol of PEP consumed to 1 mol of lactose transported via the lac-PTS, the observed accumulation of disaccharide was only 40 to 50% that expected from the PEP potential in vivo. Since the total PEP potential was consumed concomitant with maximum uptake of all sugars, the data suggested a stoichiometric ratio of ca. 2 mol of PEP utilized to 1 mol of lactose accumulated. Clearly, a considerable proportion (ca. 40%) of the PEP potential had been utilized for a purpose other than translocation of lactose via the lac-PTS. This conclusion was confirmed by data obtained from chromatography and enzymatic analysis: (i) after maximum accumulation of lactose (10 to 15 s) lac 6-P was not detectable in cell extracts, but (ii) gal 6-P, G6P, and F6P (formed from G6P via phosphohexoseisomerase) were present at high levels. Since cleavage of lac 6-P would yield gal 6-P and glucose, an explanation was required for the fact that the glucose moiety of the disaccharide was recovered mainly as G6P and not as the free sugar (Table 2). Phosphorylation of glucose by endogenous ATP was eliminated since ATP was not detected in starved cells at the commencement of lactose uptake, and the additional presence of IAA prevented ATP generation from glycolysis (Table 1). The data suggested that the PEP potential served either directly or indirectly as the phosphoryl donor for G6P formation by one (or more) of the following three possible mechanisms: (i) efflux of free glucose followed by reentry and phosphorylation by the glucose-PTS, (ii) intracellular phosphorylation via the glucose PTS, or (iii) phosphorylation by ATP produced from the PEP potential by pyruvate kinase. Kinetic considerations seem to eliminate the first proposal. Phosphorylation of phosphotransferase sugars is believed to occur simultaneously with translocation, and this viewpoint appears to weaken the argument for the second mechanism. However, Button et al. (6) demonstrated that intracellular glucose (derived from maltose) in *S. aureus* could be phosphorylated

via the glucose-PTS, and therefore the second mechanism must be seriously considered in the present context. The third proposal is equally plausible in the light of recent experiments conducted in this laboratory. These studies (40, 41) showed that maintenance of the PEP potential by starved cells of *S. lactis* ML₃ was a consequence of depletion of intracellular activators of pyruvate kinase. Conversely, in the presence of an appropriate effector for the allosteric enzyme, the PEP potential also becomes an ATP potential. Gal 6-P was one of several phosphorylated metabolites which activated pyruvate kinase in vitro (38). The formation of this compound (and perhaps lac 6-P per se) during lactose uptake may permit ATP generation from the PEP potential by "triggering" the previously inactive pyruvate kinase. Phosphorylation of the glucose moiety would then occur by an ATP-dependent hexo(gluco)kinase. It should be pointed out that ATP was not detectable in cell extracts prepared 1 s after lactose uptake (data not shown), and this would seem to strengthen the argument for the second mechanism. Alternatively, the negative result may demonstrate that ATP formed by the third mechanism was used for glucose phosphorylation as rapidly as it was formed. The "diversion" of PEP potential into phosphorylation of glucose by either the second or the third mechanism would result in reduced uptake of lactose in comparison with accumulation of the monosaccharides (Fig. 2B).

Possession of the PEP:lac-PTS, phospho- β -D-galactoside galactohydrolase, and the enzymes of the D-tagatose 6-phosphate pathway are prerequisites for rapid, homolactic fermentation of lactose by group N streptococci (25). The cleavage products from lac 6-P, namely gal 6-P and glucose, must be metabolized concurrently to triose phosphates, and this balanced metabolism requires the efficient phosphorylation of free glucose. In starved cells of *S. lactis* ML₃, approximately 60% of the total PEP potential was consumed directly in phosphorylation of the galactosyl moiety of the disaccharide, whereas the residual 40% was utilized as the phosphoryl donor for the glucose moiety. These data, obtained in vivo, demonstrate that the mechanisms responsible for phosphorylation of both hexose moieties of lactose must be closely integrated and highly efficient in their use of the available PEP potential.

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ADDENDUM IN PROOF

The [^{14}C]lactose phosphate from *S. lactis* ML₃ was cleaved to galactose phosphate plus [^{14}C]glucose by highly purified phospho- β -galactosidase prepared from *Streptococcus mutans*. The enzyme preparation was a generous gift from Robert Calmes, University of Kentucky.

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